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LncRNA SNHG6 can regulate the proliferation and apoptosis of rat degenerate nucleus pulposus cells via regulating the expression of miR-101-3p

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Abstract. – OBJECTIVE: Intervertebral disc (IVD) degeneration (IDD) is a well-known consequence of low back pain, as characterized by aberrant cell proliferation and apoptosis of nucleus pulposus (NP) cells. In the present study, we aimed to investigate the effect of lncRNA small nucleolar RNA host gene 6 (SNHG6) on deregulated functions of degenerative NP cells.

MATERIALS AND METHODS: After the establishment of rat IDD models, the mRNA and protein levels of collagen-I (Col-I) and collagen II (Col-II), and mRNA level of SNHG6 were detected by using reverse transcription quantitative Real Time-PCR (RT-qPCR) and Western blot. We further investigated the role and molecular mechanisms of SNHG6 by overexpressing or silencing it in degenerative NP cells. Cell proliferation was measured by MTT assay and EdU staning, and apoptosis was measured by flow cytometry. The target of SNHG6 was identified by starBase and Dual-Luciferase reporter assay.

RESULTS: Upregulation of SNHG6 was found in IDD NP cells than in normal cells, associated with higher level of Col-I and lower level of Col-II. Overexpression of SNHG6 inhibited cell proliferation and enhanced apoptosis, accompanied by increased expression of Bax, caspase-3, and p21, as well as decreased expression of Bcl-2, which was in reverse to the treatment of SNHG6 silencing. Moreover, miR-101-3p was indicated as a target of SNHG6, and inhibition of miR-101-3p reversed the effects on proliferation and apoptosis induced by SNHG6.

CONCLUSIONS: SNHG6 suppressed cell proliferation and induced apoptosis by increasing expression of Bax, caspase-3, p21 and decreasing Bcl-2 through targeting miR-101-3p, which suggested that SNHG6 could be a potential target in the treatment of IDD.

Key Words:
Degenerative disc disease, SNHG6, Nucleus pulposus cells, MiR-101-3p.

Introduction

Intervertebral disc (IVD) degeneration (IDD) is one of the most common causes of low back pain, creating important public health problems[1,2]. IDD is an age-related disease, which occurs in 90% of people aged over 50. The possible causes of IDD are multifactorial and a clear understanding of pathogenesis of IDD is still lacking.

The IVD is composed of the central nucleus pulposus (NP), the outer annulus fibrosus (AF), and the cartilage endplate (CEP) covering the vertebral body[3,4]. NP cells are crucial for resisting comprehensive loads and preserving IVD integrity through producing extracellular matrix (ECM) components[5-7]. However, IDD is characterized by deregulation of NP cell functions, including aberrant cell proliferation, apoptosis, and a progressive decline of ECM[8-10].

Long non-coding RNA (lncRNA) is a class of transcripts longer than 200 nucleotides without coding potential[11]. Deregulated lncRNAs are involved in various types of diseases, including cancer, autoimmune, cardiovascular, and bone diseases[12-14]. LncRNAs play key roles in regulating various cellular phenotypes, such as proliferation, apoptosis, migration and invasion[15,16]. LncRNAs function as key regulators in the development of IDD in relation to their effects on NP cell proliferation, apoptosis, and ECM synthesis[17,18]. Ln-

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cRNA-targeted therapy might be an effective measure against IDD. The lncRNA-sequence data from our recent work has identified higher expression of lncRNA small nucleolar RNA host gene 6 (SNHG6) in tissues of IDD patients as compared with control samples. However, the roles of SNHG6 on NP cells are still unexploited.

In the present study, we investigated, for the first time, the effects of SNHG6 on proliferation and apoptosis of NP cells isolated from rat IDD models and illustrated the possible mechanisms. These findings highlight the potential of a novel target for IDD therapy.

Materials and Methods

Rat Intervertebral Disc Degeneration Model

Wistar rats were purchased from Medical College of Southeast University (Nanjing, China) and kept in a well-ventilated and quiet animal house of Zhongda Hospital, Southeast University for one week of proper acclimation prior to the experiments. Rat intervertebral disc degeneration (IDD) model was created using the puncture method described by Issy et al[19]. Rats were anesthetized with 3% sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA) (40 mg/kg injected intraperitoneally). Then, the needle (21G) puncture was performed at levels of lumbar intervertebral disc L3-4 according to the X-ray images. Two weeks later, the extents of disc degeneration were determined using MRI. All experiments conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and all the protocols were approved by the Animal Care and Use Committee of Zhongda Hospital, Southeast University.

Isolation of Degenerative Nucleus Pulposus Cells

Rat nucleus pulposus (NP) cells were isolated from the L3-4 lumbar intervertebral discs with the method described by Hiyama et al[20]. Tissues were separated from lumbar discs, minced into small fragments, and digested using 0.25% trypsin (Thermo-Fisher Scientific, Waltham, MA, USA) at 37°C. The samples were centrifuged and the sediment was resuspended in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), and 1% (w/v) penicillin/streptomycin (Gibco) and maintained at 37°C in a humidified atmosphere of 5% CO_2. The unattached NP cells were collected and transferred into 10 cm dishes. Low passages (1 to 3) of cells were used for our experiments. Protein and mRNA levels of collagen I and collagen II, the specific indicators for degenerative and normal NP cells[8,21] were analyzed.

Cell Treatment

SNHG6 overexpression plasmid pcDNA3.1-SNHG6 as well as SNHG6 siRNA were purchased from RiboBio (Guangzhou, China). miR-101-3p mimics, miR-101-3p NC and miR-101-3p inhibitors were purchased from Genepharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as the transfection reagent. The expression of SNHG and miR-101-3p was detected by RT-qPCR.

Cell Viability

Cell viability was measured using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Bio-Rad, Hercules, CA, USA). Cells of each group were seeded into a 96-well culture plate (Thermo Fisher Scientific) at the density of 1×10^3 cells/well and cultured for different periods of time (0, 12, 24 and 48 h). Following the manufacturer's protocol, 10 μL of MTT reagent were added to each well. After incubation for 4 h at 37°C, the medium was discarded, and the cells were incubated with 150 μL of DMSO (Bio-Rad). The plate was shaken on a microvibrator for 10 min and the optical density (OD) of each well was measured using an ELISA reader (Bio-Rad) at 490 nm.

EdU (5-Ethynyl-29-Deoxyuridine) Labeling

Cells were planted into a 12-well plate and recovered overnight in a 95% humidified incubator at 37°C with 5% CO_2, followed by serum starvation for 24 h in the same condition. Afterwards, cells were exposed to serum for 24 h, during which cells were treated with EdU (20 μM) from the Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies, Carlsbad, CA, USA) at the last 3 h. After then, cells were fixed and permeabilized by 4% PFA and 0.5% Triton-X, followed by incubation with Click-iT reaction cocktail (including $CuSO_4$ and Alexa Fluor Azide), and nuclear staining by DAPI. Images were captured by an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany), data were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell Apoptosis Assay

The apoptotic cells were measured using Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's instructions. Cells were washed with ice-cold phosphate-buffered saline (PBS), and re-suspended in 1 × cold binding buffer. Next, cells were stained with 5 μL of Annexin V/FITC and 10 μL of PI for 15 min at room temperature in the darkness. The stained cells were subjected to a flow cytometry (BD Biosciences) and analyzed by CellQuest software (BD Biosciences).

Western Blot Assay

Total protein extracts of each culture were prepared using ice-cold lysis buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland). The cell lysates were used for electrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), then transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Darmstadt, Germany). The membranes were blocked in Tris buffered saline Tween 20 (TBST) solution containing 5% skim milk at room temperature for 2 h, and then probed overnight with primary antibodies at 4°C. After washing and incubating with horseradish peroxidase (HRP)-conjugated secondary antibody, immunoreactive proteins were visualized with the SuperSignal® West Pico kit (Pierce, Rockford, IL, USA). The intensity of the target bands was quantified using Image J software (National Institutes of Health) and normalized against GAPDH and the untreated control.

Reverse Transcription Quantitative Real-time PCR (RT-qPCR)

Total RNA isolation was performed using TRIzol reagent (Invitrogen) and converted into cDNA using PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). PCR reactions were performed in triplicate using SYBR® Premix Ex Taq™ kit (TaKaRa, Dalian, China) with the gene-specific primers. The following sequences of primers were used: SNHG6 forward (5'-3'): CCTACTGACAACATCGACGTTGAAG, reverse: GGAGAAAACGCTTAGCCATACAG; Col-I forward: TGACCTCAAGATGTGCCACT, reverse: ACCAGACATGCCTCTTGTCC; Col-II forward: GGTGACTACTGGATAGACCC, reverse: TGAAGTGGAAGCCGCCA; p21 forward: GGCTGTCTCCGCTCATAG, reverse: CAGCAGACCACCATTTCA; Bcl2 forward: AGATGTCCAGCCAGCTGCAC, reverse: TGTTGACTTCACTTGTGGCC; Bax forward: ACCAAGAAGCTGAGCGAGTGT, reverse: ACAAACATGGTCACGGTCTGC; caspase-3 forward: CTCGGTCTGGTACAGATGTCGATG, reverse: GGTTAACCCGGGTAAGAATGTGCA; GAPDH forward: GGGAGCCAAAAGGGTCAT, reverse: GAGTCCTTCCACGATACCAA; miR-101-3p forward: UACAGUACUGUGAUAACUGAA, reverse: CAGUUAUCACAGUACUGUAUU; U6 forward: GCUUCGGCAGCACAUAUACUAAAAU, reverse: CGCUUCACGAAUUUGCGUGUCAU. The relative expression levels of LncRNA and genes were calculated by comparison with the internal control GAPDH using the $2^{-\Delta\Delta Ct}$ method. The expression of miR-101-3p was normalized to U6 using the $2^{-\Delta\Delta Ct}$ method.

Bioinformatics, Plasmid Construction, and Dual-Luciferase Reporter Assay

The target miRNA of SNHG6 was predicted by StarBase Version 3.0. Plasmid containing the sequences at 3'-UTR of MIAT paring the miR-101-3p (MIAT-WT) and the mutant 3'-UTR regions (MIAT-MUT) was synthesized by GenePharma (Shanghai). The plasmid was then transfected into 293T cells, and co-transfected with miR-101-3p mimics or miR-101-3p NC. 48 h after transfection, the activity of the Luciferase was examined using the Dual-Luciferase reporter assay kit (Beyotime, Shanghai, China).

Statistical Analysis

All data were analyzed using the GraphPad Prism 5 software (La Jolla, CA, USA) and expressed as mean ± SD. Comparisons between two groups were analyzed by unpaired Student's *t*-test. ANOVA followed by Newman-Keuls test was applied to evaluate the difference among multigroups. $p<0.05$ was considered statistically significant.

Results

Degenerative NP Cells Shows ECM Degradation

NP cells were isolated from healthy and IDD rats. The expression levels of ECM-degrading molecule Col-I and ECM-coding molecule Col-II[22] were measured using RT-qPCR and Western blot assays. As shown in Figure 1A and B, a significant increase in Col-I expression and a remarkable decrease in Col-II expression was observed in degenerative NP cells ($p<0.01$). Similarly, as compared to the normal cells, the mRNA level of Col-I was upregulated and Col-II was

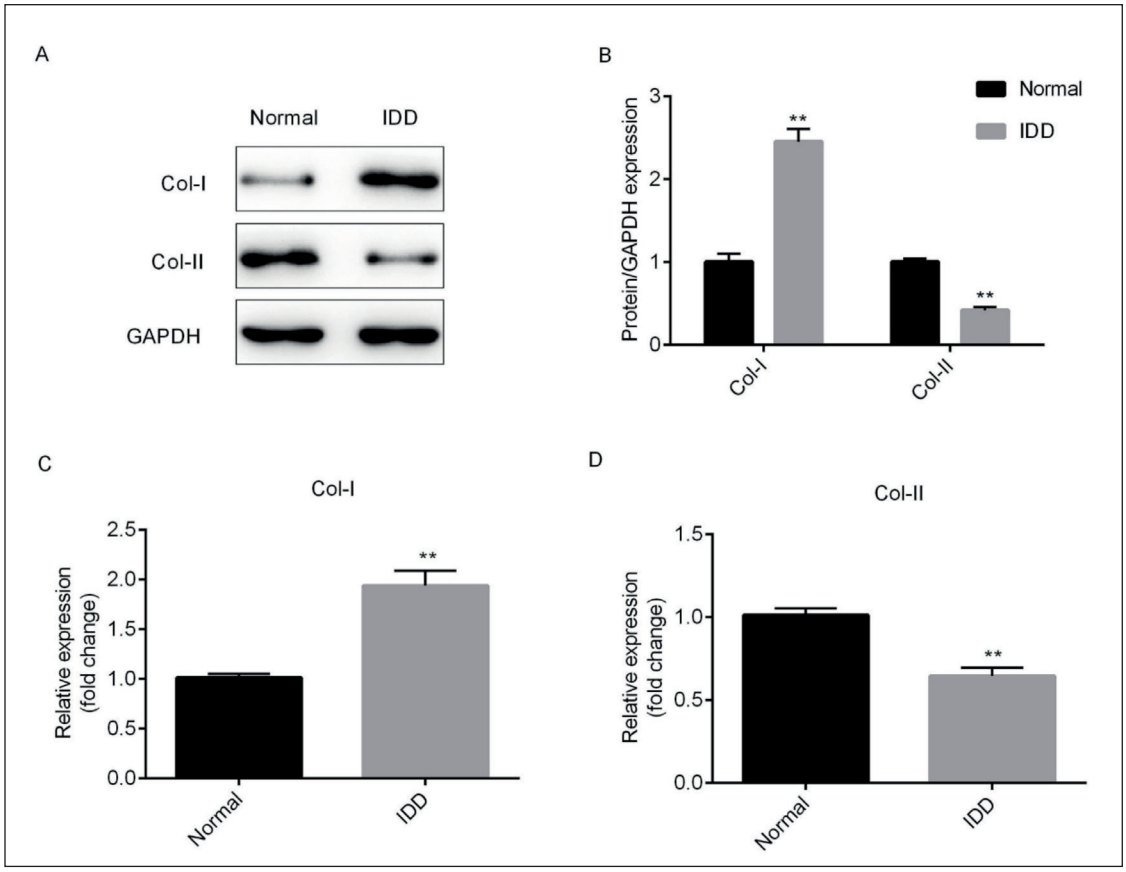

Figure 1. Expression levels of Col-I and Col-II in NP cells from normal and IDD rats. **A**, Western blot was conducted for analysis of Col-I and Col-II. The representative image is shown. GAPDH was used as the internal control. **B**, Quantitative analysis of the protein expression of Col-I and Col-II was performed. RT-qPCR was conducted for analysis of (**C**) Col-I and (**D**) Col-II. Data were normalized with the level of GAPDH control. Results were expressed relative to the value of controls (set at 1.0 as the reference). $^{**}p<0.01$.

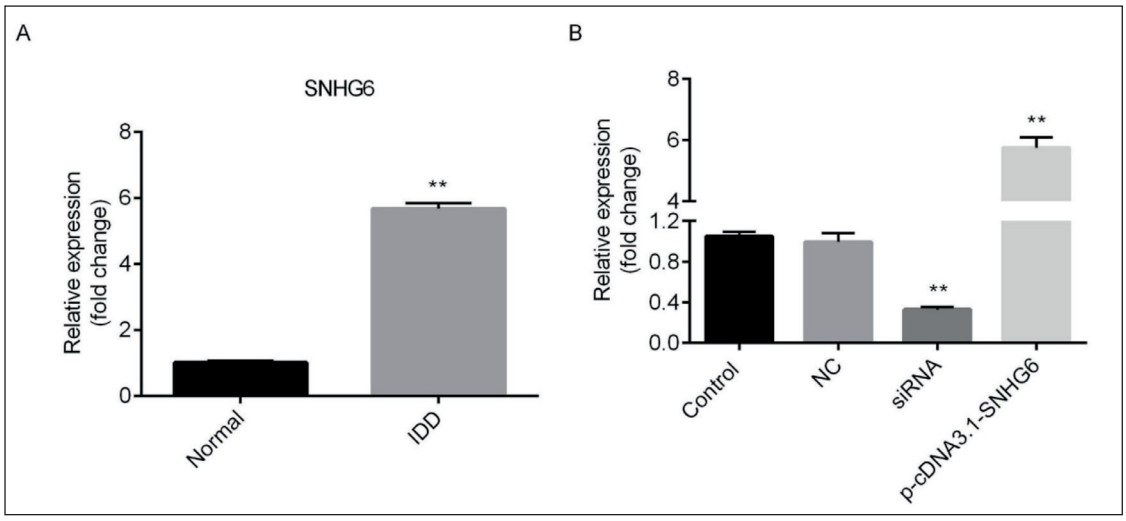

Figure 2. mRNA level of SNHG6 in NP cells. **A**, RT-qPCR was conducted for analysis of SNHG6 in NP cells from normal and IDD rats. **AB**, RT-qPCR was conducted for analysis of SNHG6 after degenerative NP cells transfected with pcDNA3.1-SNHG6 and SNHG6 siRNA. Data were normalized with the level of GAPDH control. Results were expressed relative to the value of controls (set at 1.0 as the reference). $^{**}p<0.01$.

downregulated in degenerative NP cells (Figure 1C and D, $p<0.01$).

Expression of SNHG6 in Degenerative NP Cells

As shown in Figure 2A, degenerative NP cells showed upregulated expression of SNHG6 relative to normal controls. To investigate the biological effects of SNHG6, degenerative NP cells were transfected with pcDNA3.1-SNHG6, as well as SNHG6 siRNA. As shown in Figure 2B, SNHG6 level was found to be significantly lower in SNHG6 siRNA-transfected cells, and markedly higher in pcDNA3.1-SNHG6 transfected cells, compared with control.

Effects of SNHG6 on Cell Viability and Apoptosis of Degenerative NP Cells

SNHG6 overexpression significantly decreased cell viability of degenerative NP cells in a time-dependent manner compared with control or empty vector group (Figure 3). As expected, results of MTT and EdU staining assays showed that SNHG6 knockdown remarkably suppressed cell viability over the same time period, the significant differences were observed at 48 h post transfection (Figure 3A and B, $p<0.01$). On the other hand, significant induction of apoptosis was observed with SNHG6 overexpression in degenerative NP cells. However, SNHG6 knockdown markedly suppressed apoptosis of cells (Figure 4A and B, $p<0.01$).

Effects of SNHG6 on Apoptosis-Related Molecules

We further analyzed the possible mechanisms underlying SNHG6-mediated cellular responses. RT-qPCR results demonstrated that overexpression of SNHG6 increased the expression of pro-apoptotic proteins Bax and caspase-3, anti-proliferative protein p21, but reduced the expression of anti-apoptotic protein Bcl-2, while salience of SNHG6 have the opposite effects on these factors (Figure 5, $p<0.01$). Similar effects were noted in protein expression levels of p21, Bcl-2, Bax and caspase-3, which was measured by Western blot (Figure 6, $p<0.01$).

MiR-101-3p is a Target of SNHG6

LncRNAs contain the region with the sequences complementary to miRNAs, and some previous studies suggested LncRNAs may exert their functions *via* targeting miRNAs. We performed bioinformatic analysis using starBase Version 3.0 database, and miRNA-101-3p (miR-101-3p) was found as a target of LncRNA SNHG6 (Figure 7A). To further indicate the relationship between SNHG6 and miR-101-3p in IDD, RT-qPCR has performed to examine the expression of miR-101-3p in degenerative NP cells and normal cells. It was observed that the expression level of miR-101-3p was significantly down-regulated in degenerative NP cells compared with the normal cells (Figure 7B, $p<0.01$), and the expression level of SNHG6 was negatively correlated with the expression of miR-101-3p (Figure 7C, r=-0.7908, $p=0.0022$). Moreover, the expression of miR-101-3p was markedly increased in by SNHG6 siRNA

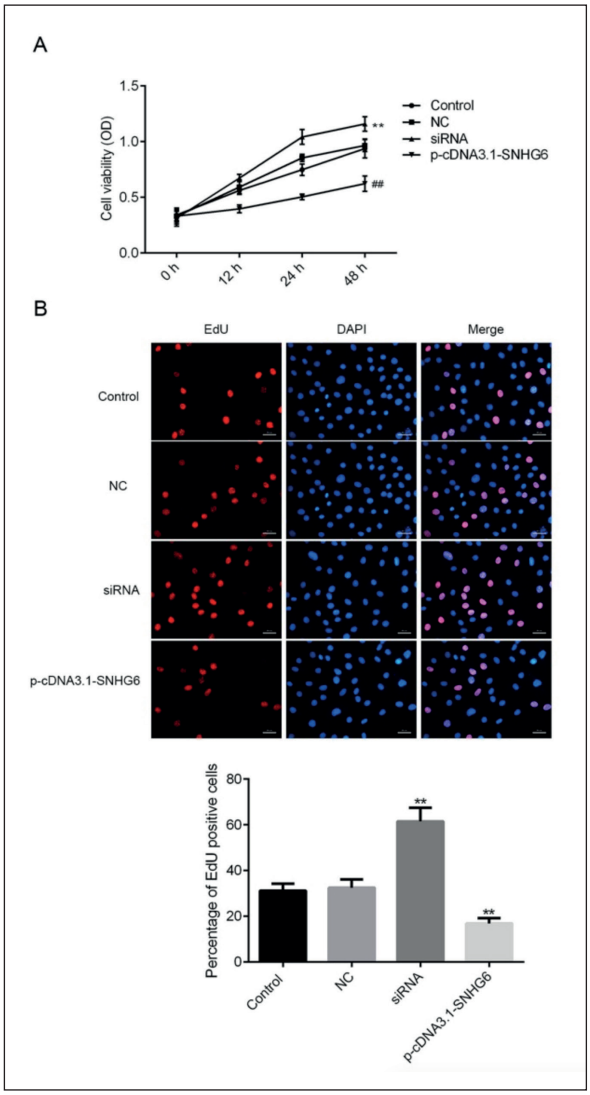

Figure 3. Effects of SNHG6 on cell viability. **A**, MTT assay was undertaken to detect cell viability. **B**, EdU assay was undertaken to detect cell viability (magnification: ×50). The data were shown as the optical density measured at 490 nm. $^{**}p<0.01$.

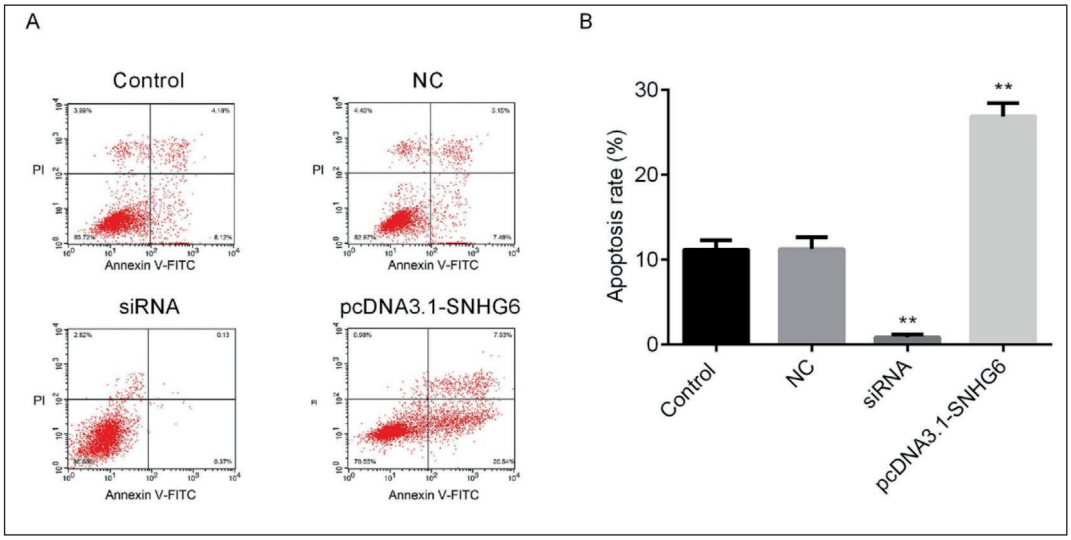

Figure 4. Effects of SNHG6 on cell apoptosis. Cell apoptosis was assessed using flow cytometry following Annexin V-FITC/PI staining. **A**, Representative scatter plots are shown. **B**, Apoptotic rates were calculated and presented as mean ± SD from three separate experiments. $^{**}p<0.01$.

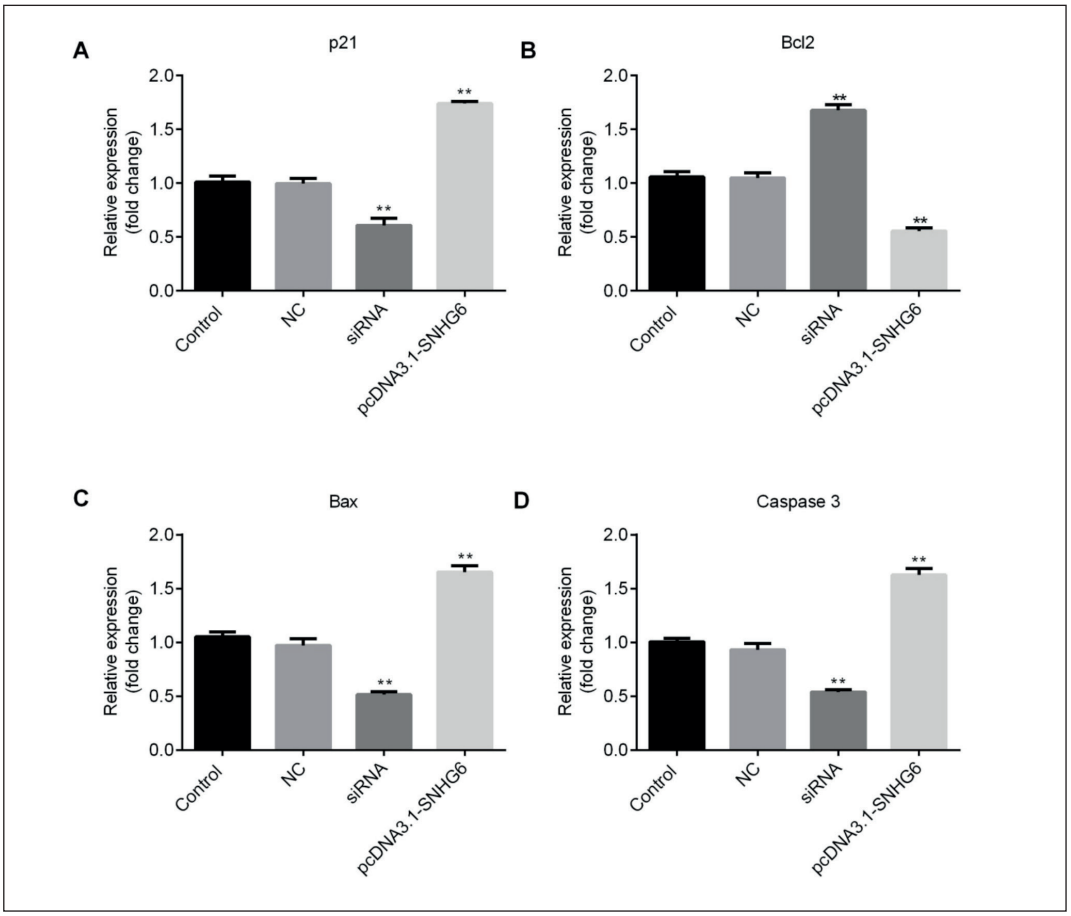

Figure 5. Effects of SNHG6 on the mRNA expression apoptosis-related molecules. RT-qPCR was conducted for analysis of (**A**) p21, (**B**) Bcl-2, (**C**) Bax and (**D**) caspase-3. Data were normalized with the level of GAPDH control. Results were expressed relative to the value of controls (set at 1.0 as the reference). $^{**}p<0.01$.

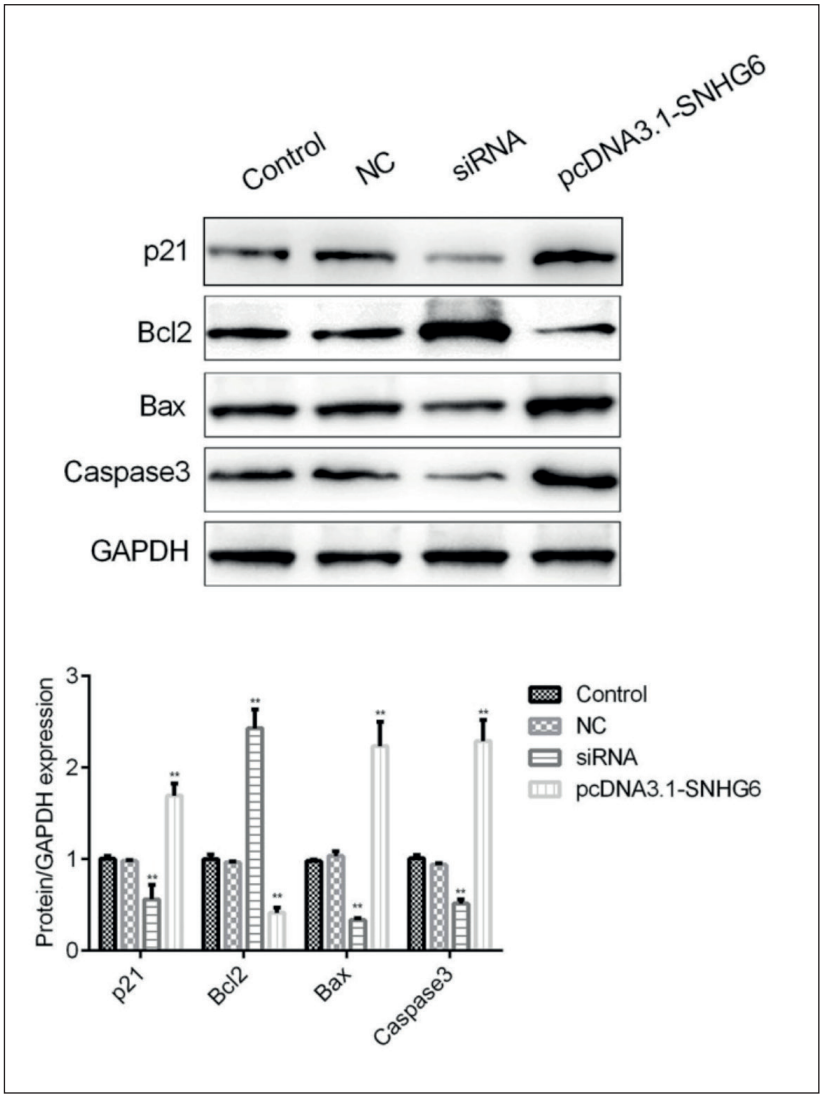

Figure 6. Effects of SNHG6 on the protein expression apoptosis-related molecules. **A**, Western blot was conducted for analysis of Bax, Bcl-2, caspase-3, and p21. The representative image is shown. GAPDH was used as the internal control. **B**, Quantitative analysis of expression of the indicated proteins was performed. $^{**}p<0.01$.

(Figure 7D, $p<0.001$). Next, the direct targeting relationship between SNHG6 and miR-101-3p was further confirmed by dual-luciferase reporter assay. It was observed that the activity of Luciferase in SNHG6 WT group was significantly decreased by the treatment of miR-101-3p mimics ($p<0.01$), while there was no significant effect on Luciferase activity in co-transfection of miR-101-3p mimics and SNHG6 MT group (Figure 7E, $p<0.01$). These results suggested that SNHG6 may be involved in the pathogenesis of IDD via regulating the expression of miR-101-3p.

SNHG6 Can Regulate the Proliferation and Apoptosis of NP Cells Via Targeting MiR-101-3p

Finally, to determine whether SNHG6 can regulate the proliferation and apoptosis of NP cells *via* targeting miR-101-3p, NP cells were transfected with SNHG6 siRNA and treated with or without miR-101-3p inhibitor, and the proliferation and apoptosis of the cells was examined. As shown in Figure 8, treatment of miR-101-3p inhibitor partially blocked SNHG6 siRNA induced pro-proliferative effects (Figure 8A and B, $p<0.01$). On the other hand, miR-101-3p inhibitor also partially abrogated SNHG6 siRNA induced anti-apoptotic effects (Figure 9, $p<0.05$)

Discussion

LncRNAs play crucial roles in bone diseases, such as osteoarthritis and osteosarcoma[23,24]. In particular, an increasing number of studies

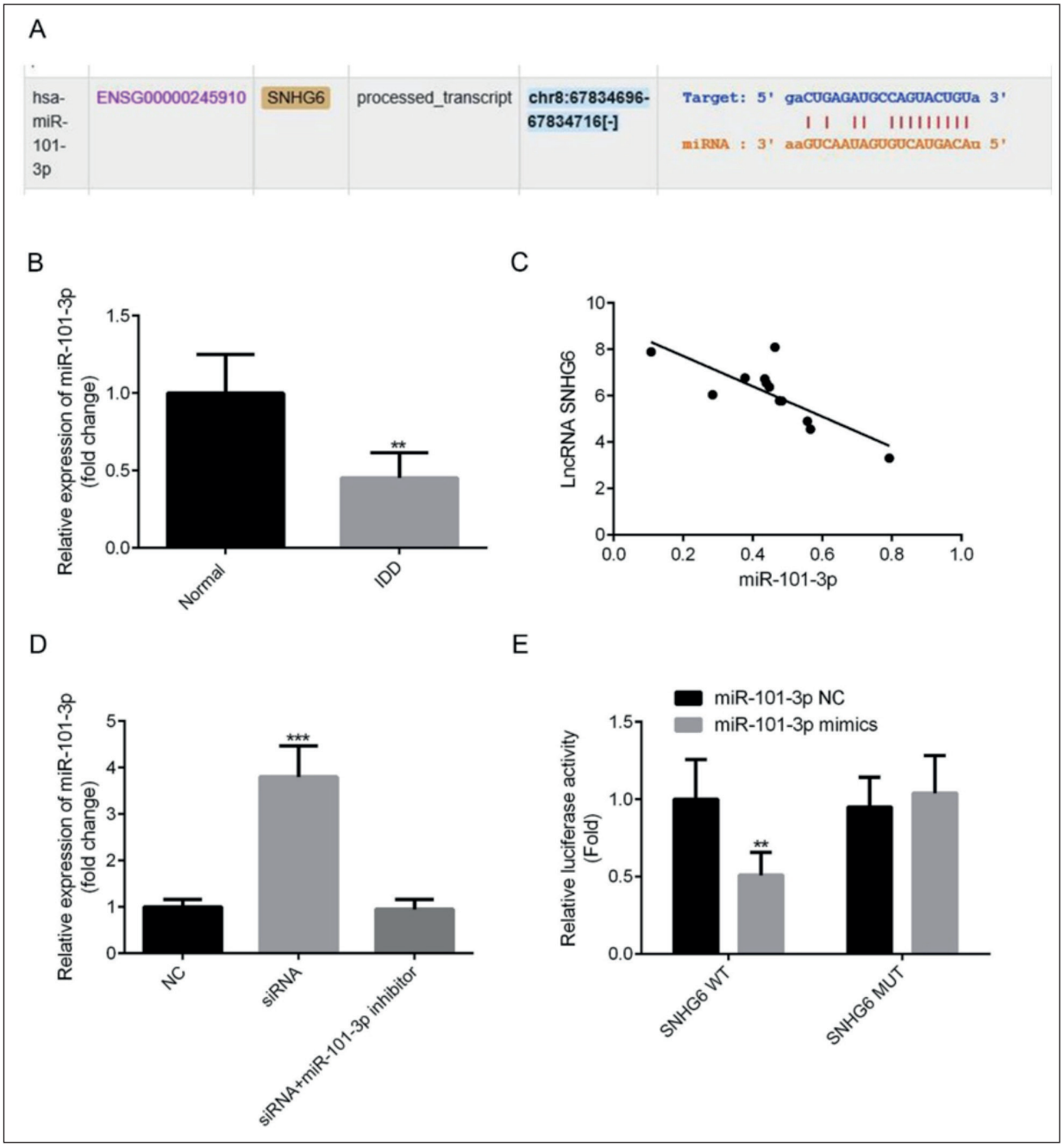

Figure 7. miR-101-3p is a target of SNHG6. **A**, The paring 3'-UTR region of SNHG6 and miR-101-3p. **B**, Relative expression of comparison of the expression of miR-101-3p in degenerated NP cells and the normal NP cells. **C**, The correlation between the expression of SNHG6 and miR-101-3p in degenerated NP cells. **D**, Effect of SNHG6 siRNA on the expression of miR-101-3p in degenerated NP cells. **E**, Results of the dual-luciferase reporter assay. $^{**}p<0.01$, $^{***}p<0.001$.

have supported the functional roles of lncRNAs in IDD. In the present study, we evaluated expression level of lncRNA SNHG6 in NP cells of IDD rats. SNHG6 was significantly upregulated in degenerative NP cells compared with normal controls. Its higher expression was associated with ECM degradation of degenerative NP cells, which indicates that SNHG6 might affect NP cell functions.

Cellular mechanisms of NP deregulation contributing to IDD involve in aberrant cell proliferation and apoptosis. To illustrate the roles of

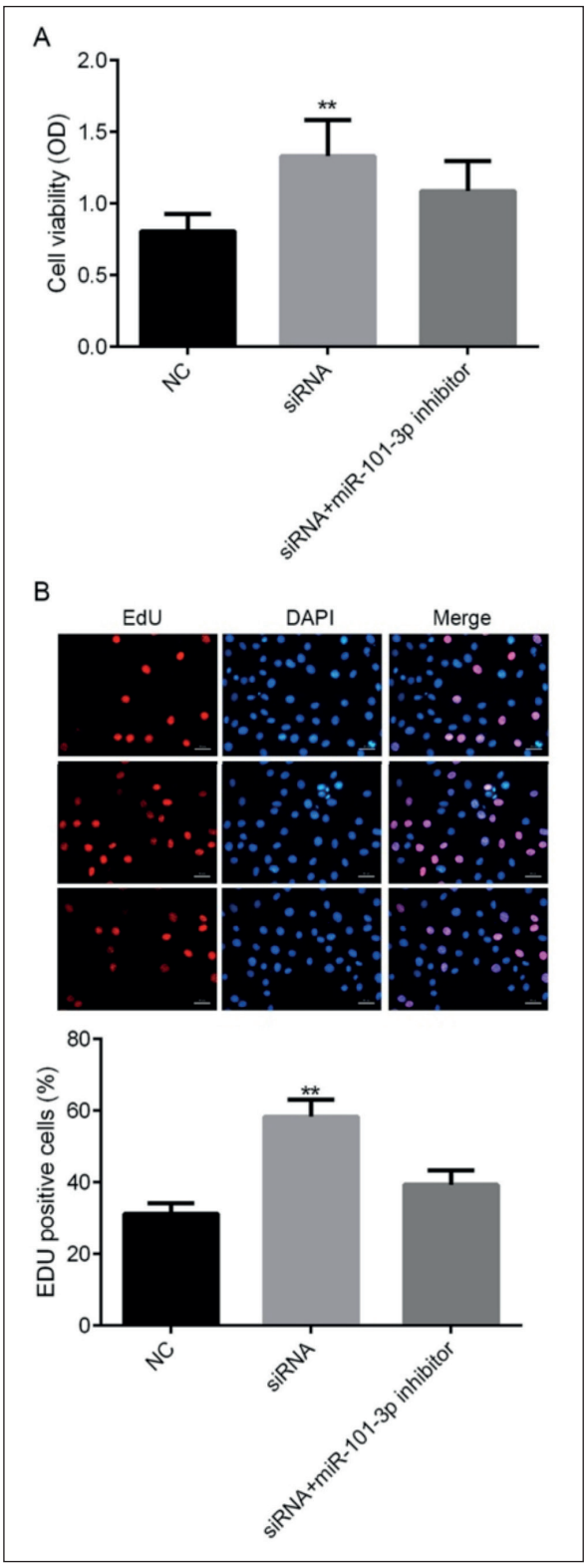

Figure 8. SNHG6 can regulate the proliferation of NP cells via targeting miR-101-3p. **A**, MTT assay was undertaken to detect cell viability. The data were shown as the optical density measured at 490 nm. **B**, EdU assay was undertaken to detect cell viability (magnification: ×50). $^{**}p<0.01$.

SNHG6 in NP cell degeneration, degenerative NP cells were transfected with SNHG6 siRNA or pcDNA3.1-SNHG6. Our data showed that SNHG6 overexpression can cause decreased cell viability and increased apoptosis, whereas these phenomena can be reversed by SNHG6 knockdown.

p21, encoded by CDKN1A, is a potent cyclin-dependent kinase inhibitor. It can prevent cell cycle progression *via* binding to and inhibiting CDK2 activity[25,26]. p21 has been implicated in the regulation of SNHG6 in cancers[25,27] and IDD pathology[28,29]. Herein, overexpression or silencing of SNHG6 affect cell viability might partially dependent on p21 expression. Bax and Bcl-2 are pro-apoptotic and anti-apoptotic proteins respectively, and the high ratio of Bax/Bcl-2 has been demonstrated to be linked with increased apoptosis rate[30,31]. In the present study, overexpressing or silencing SNHG6 caused high or reduced ratio of Bax/Bcl-2. Moreover, SNHG6-induced caspase-3 expression indicated a caspase-dependent apoptosis, which was suppressed by SNHG6 silencing.

LncRNAs can exert their function *via* various mechanisms, among which the interaction between LncRNA and miRNAs is the most commonly observed mechanism[32,33]. Using bioinformatic methods, we found that miR-101-3p is a target of SNHG6 and SNHG6 can negatively regulate its expression. SNHG6 targets miR-101-3p to promote tumorigenesis in several cancers[34,35]. Moreover, through bioinformatic tool TargetScan, there are more than 2000 target genes predicted to be targeted by miR-101-3p. In the present study, we found that miR-101-3p was downregulated in degenerative NP cells compared with normal NP cells. Deletion of SNHG6 upregulated miR-101-3p level, which was reduced by inhibition of miR-101-3p. Moreover, SNHG6 can regulate the proliferation and apoptosis of NP cells *via* regulating the expression of miR-101-3p. These results suggested that that elevated SNHG6 may facilitate the development of IDD *via* downregulating the expression of miR-101-3p in NP cells and lead to the abnormal behaviors of the cells.

Conclusions

Briefly our study demonstrated that SNHG6 might promote IDD development partially by inhibiting proliferation and promoting apoptosis of NP cells by targeting miR-101-3p. These findings support that SNHG6 serves as a potential therapeutic target for IDD therapy.

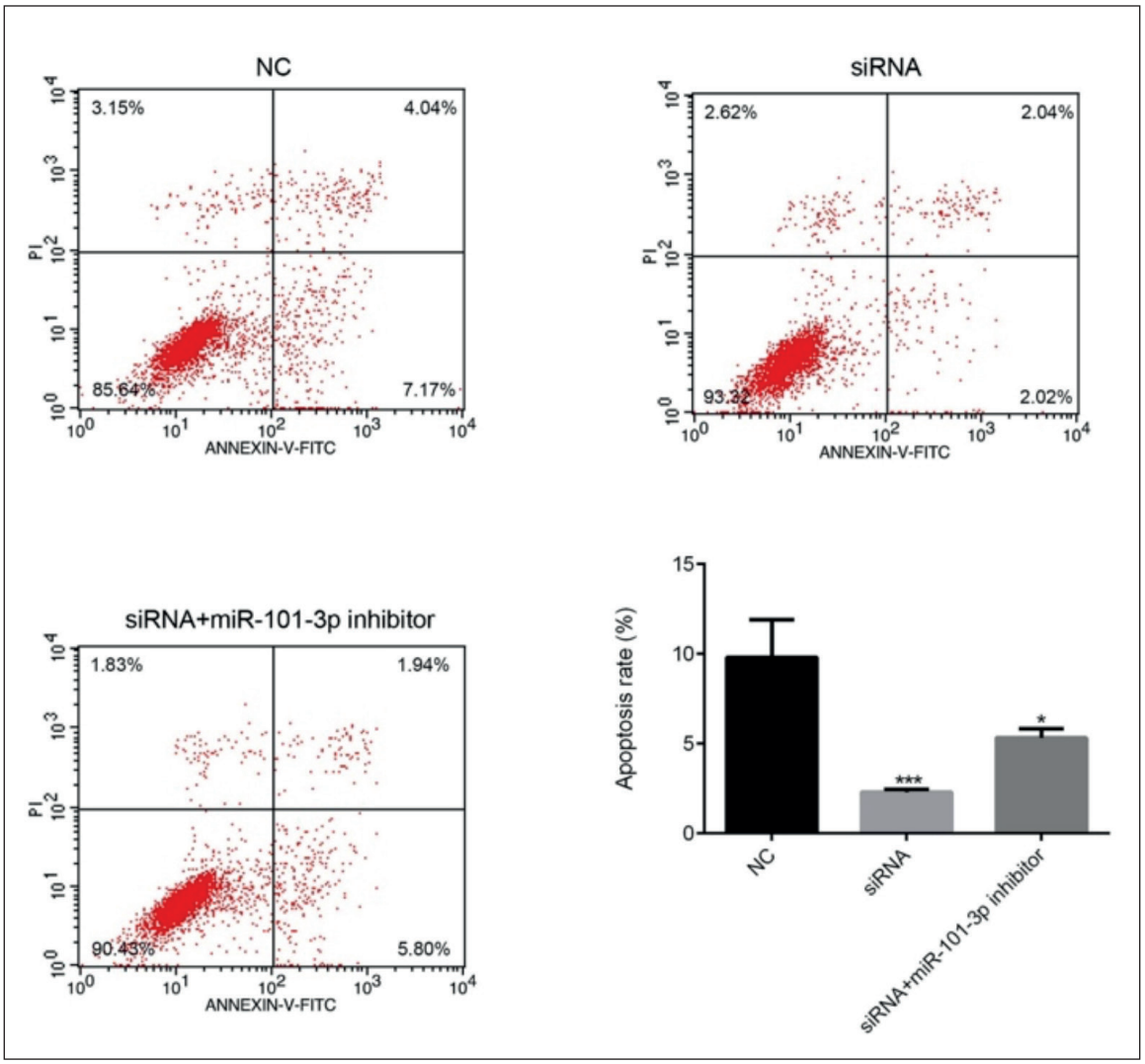

Figure 9. SNHG6 can regulate the apoptosis of NP cells via targeting miR-101-3p. Flow cytometry assay was undertaken to detect cell apoptosis. *p<0.05 and ***p<0.001.

Conflict of Interests

The authors declare that they have no conflict of interests.

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